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IDENTIFICATION AND CHARACTERIZATION OF MIXED DISULPHIDE COMPLEXES OF E APOPROTEIN IN HIGH DENSITY LIPOPROTEIN OF SUBJECTS WITH ACUTE ALCOHOLIC HEPATITIS

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Summary: Two E apoprotein complexes have been isolated from the plasma high density lipoprotein fraction of patients with alcoholic hepatitis. Both were mixed disulphide complexes and could be dissociated into subunits with β-mercaptoethanol but not with 1% sodium dodecyl sulphate or 8 M urea. One of the complexes (molecular weight 46,000) was identified as an E-AII unit and the other, of molecular weight 106,000 was consistent in properties with an E trimer. The latter has not been described before and the E-AII complex has been reported once previously in plasma of patients with Type III hyperlipoproteinaemia. It is proposed that the presence of E complexes in abnormal disease states may affect the normal recognition of E monomer by cells thereby altering the subsequent fate of their host lipoproteins and their lipid constituents.

INTRODUCTION

Human alcoholic hepatitis is associated with marked changes in the concentration and composition of plasma lipoproteins (1). The abnormal high density lipoprotein (HDL) is characterized by relative small amounts of AI and high concentrations of E apoproteins, the near absence of cholesteryl ester (2) and occurrence of stacked discoidal particles. The striking similarity of this abnormal HDL, both in composition and ultrastructure to newly secreted HDL isolated from rat liver perfusates has led to the suggestion (2) that the abnormal HDL in this disease may be nascent HDL.

In addition to E apoprotein, other unidentified, higher molecular weight (MW) proteins have been reported (2). This communication describes the isolation and characterization of two of these proteins as complex forms of the E apoprotein.

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MATERIALS AND METHODS

Experimental procedures

Plasma was obtained from subjects aged 39-51 years showing characteristic clinical, biochemical and histological features of severe alcoholic hepatitis (AH). 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and iodacetic acid treated plasma samples were obtained by adding DTNB and iodoacetic acid reagent to freshly collected blood as described by Weisgraber and Mahley (3). Lipoproteins were separated by sequential preparative ultracentrifugation in a type 40 or type 50 titanium rotor in a Beckman L5-65 centrifuge at 4°C. VLDL was isolated at d 1.006 g/ml; LDL was isolated between d 1.019-1.063 and HDL between d 1.063-1.21 g/ml (4). Lipoproteins were washed by one further centrifugation at the appropriate upper density, and then dialyzed against 5 mM NH_4HCO_3, pH 8.0.

Characterization of apoproteins

Lipoproteins in 5 mM NH_4HCO_3, pH 8.0 were lyophilized and then delipidated with chloroform, methanol and ether as described previously (5). SDS-gel electrophoresis was performed on 10 or 15% polyacrylamide gels containing 0.1% SDS according to the method of Weber and Osborn (6). Reduction of disulfide bonds was achieved by adding fresh β-mercaptoethanol (Sigma) to the samples. Molecular weight determinations of the proteins were carried out on SDS-gels (10%) and SDS-gradient gels (7) using appropriate MW markers (non-enzymatic protein MW markers, Schwarz/Mann (N.H.) and both high and low MW calibration kits (Pharmacia).

Isolation and identification of apoproteins

The E apoprotein and other high MW peptides present in HDL of AH plasma were isolated by preparative SDS-polyacrylamide gel electrophoresis on 10% gels. Protein bands were located, sliced and eluted as described before (8). Apoproteins were characterized by immunological identification, using monospecific antisera to pure apoproteins isolated from normal lipoproteins in this laboratory. AI and AII antisera were prepared by techniques described previously (8). E antisera was prepared against E apoprotein which was isolated from VLDL obtained from normal blood bank volunteers. VLDL apoproteins were separated on Sephacryl S 300 columns (Pharmacia) 2.6 x 180 cm, in 6 M urea, 0.05 M Tris-HCl, pH 8.2 and E apoprotein rich fractions applied to DEAE Sephacel (Pharmacia) columns (0.9 x 40 cm) which were eluted with a gradient ranging from 0.01 M to 0.15 M Tris-HCl, pH 8.2. All buffers contained deionized 6 M urea. The E apoprotein thus purified was identical in amino acid composition, electrophoretic mobility and MW to that described elsewhere by various groups (3,9). Immunochemical assays were performed using both double immunodiffusion and crossed immunoelectrophoresis. In the latter procedure, electrophoresis of the reduced E-AII complex in the first dimension was performed on SDS-15% polyacrylamide gels. A thin (2 mm) slice was then imbedded into 1% agarose and in the second dimension, sequentially separated by electrophoresis through 1% agarose-1% Triton X-100 and then into agarose

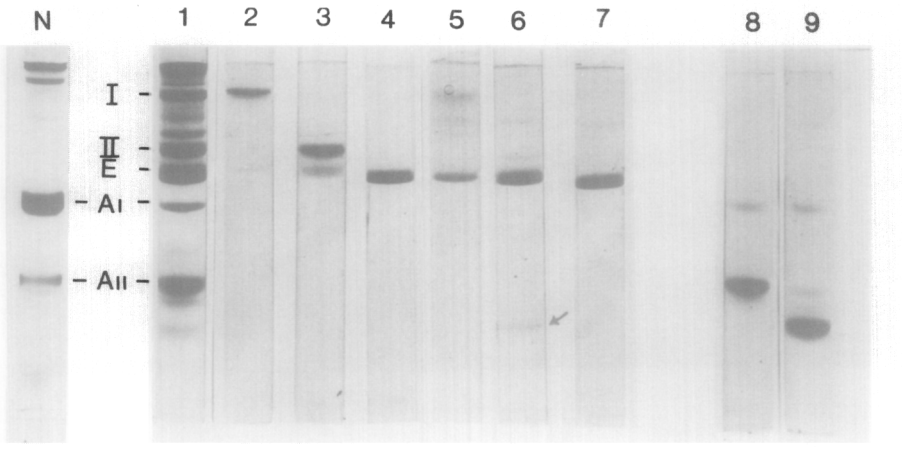

Fig. 1

Separation of apoproteins of N (normal HDL) and 1, HDL from alcoholic hepatitis patient. Gels 2,3 and 4 are proteins I, II and E isolated by preparative gel electrophoresis respectively in absence of mercaptoethanol. Gels 5,6 and 7 are I, II and E run with mercaptoethanol. Arrow points to AII apoprotein. Gels 8 and 9 are AII apoprotein not reduced and reduced respectively.

containing antibody (10). The procedure removes SDS by forming Triton X-100/SDS complexes preventing non specific precipitation of antibody by SDS.

RESULTS

Fig. 1 compares the apoprotein of HDL from normal subjects with that of a typical AH subject. The prominent AI band seen on SDS-polyacrylamide gels of normal HDL is virtually absent from the AH HDL while the latter contains E apoprotein plus other prominent high MW bands, labelled I, II (gel 1). Following reduction of these apoproteins with β-mercaptoethanol, two of the higher MW bands, I and II, virtually disappeared with apparent increases in the E apoprotein band (gels 5 and 6). In order to identify the major bands in AH HDL, the apoprotein was separated by preparative SDS gel electrophoresis (10% gels) and the protein of each band eluted and characterized on analytical SDS-gels (Fig. 1) and by immunochemical methods

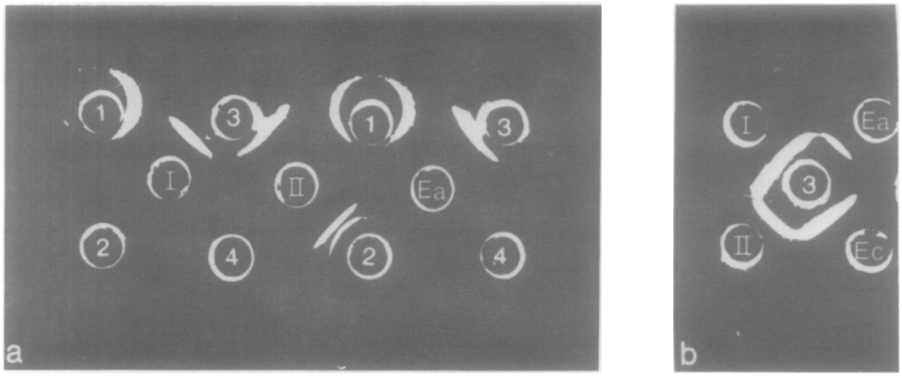

Fig. 2

(a) Immunodiffusion in 1% agarose of proteins I, II and E. Ea is E apoprotein from alcoholic hepatitis subjects. In wells numbered 1-4 are antisera to AI, AII, E and CII respectively.

(b) Immunodiffusion as for 2(a) except that Ec represents apoprotein E from control subjects.

(Figs. 2 and 3). The purity of the eluted bands I, II and E are shown in Fig. 1 (gels 2-4). Their MW (from SDS-gradient gels) were estimated to be 106,000, 46,000 and 36,000 respectively. Reduction of band II (ca 46,000) produced two subunits, the major component comigrating with E and another weaker band in the AII (monomer) position (gel 6). This protein (II) was therefore tentatively identified as the E-AII complex, since it resembled the properties of a similar complex recently characterized by Weisgraber and Mahley (3) and designated E-AII. The other high MW protein (I) produced only one band after reduction, which migrated in the same position as E apoprotein, suggesting the existence of another polymeric form of E apoprotein, not previously described. The estimated MW of 106,000 and the absence of proteins other than E apoprotein suggest the presence of an E trimer.

The identification of both the E-AII complex and the E trimer was confirmed by immunodiffusion. In Fig. 2a it can

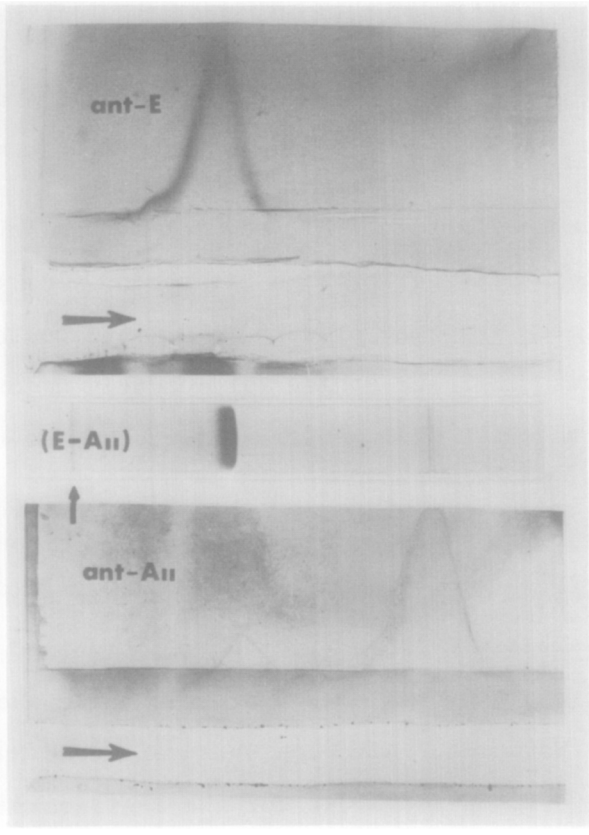

Fig. 3

Crossed immunoelectrophoresis of E-AII complex after reduction with mercaptoethanol. Horizontal arrows point to SDS gel slices imbedded in agarose containing 1% Triton X-100 and separately run in the second dimension containing either E antiserum (top) or AII antiserum (bottom). Precipitation rockets against AII are just visible near the AII and E regions respectively. A stained gel in the centre shows the position of E and AII proteins respectively.

be seen that I, II and E apoprotein reacted against E antisera, but not against AI or CII antisera. Only II (E-AII) reacted also against anti-AII. Neither reacted against anti-albumin. Other criteria for confirmation of immunochemical identity was shown by the complete fusion of the precipitin arcs of purified E and the E-AII and E trimer complexes (Fig. 2b). Additionally the identification of both E and AII

components in the E-AII complex was confirmed by crossed immunoelectrophoresis (Fig. 3).

DISCUSSION

The observation that an E-AII complex occurs in the HDL of alcoholic hepatitis patients is the second time that this E complex has been reported present in human lipoprotein fractions. Weisgraber and Mahley (3) recently described its occurrence in the VLDL of Type III subjects and as a minor constituent of VLDL and HDL-I in normal subjects. In AH patients however the E-AII complex is a major component. Scanning of stained gels (using an E apoprotein standard curve for quantitation) showed that E-AII comprised approximately 14-18% of the total AH HDL protein while about 25% was present as the E trimer. Although Weisgraber and Mahley (3) did not give quantitative values for the E-AII complex, their gel pictures indicate that the proportion was much lower than that observed in our gels of AH HDL.

The isolation and identification of the E trimer apoprotein is the first report, to our knowledge, of this complex from human lipoproteins though Ragland et al (2) noted the presence of one or more unidentified proteins of higher molecular weight, not usually seen in HDL, in their preparations. The E trimer which could not be dissociated with urea or SDS alone and required mercaptoethanol to dissociate it into E subunits, indicated that, like the E-AII complex, the E trimer was linked by interchain disulfide bands. The same apoprotein pattern was found when DTNB or iodoacetic acid was immediately added to blood, suggesting that disulfide formation was not an artifact of isolation.

The formation of E trimer must involve disulfide linkage formation between E apoprotein molecules only; although some amino acid composition analyses published previously (11,12) have reported the absence of half-cystine in E, more recent analyses of performic acid oxidised E apoprotein (3) have given values up to 2 mol ½-cystine/mol of protein, showing that disulfide interchain reaction between E proteins is possible.

The interesting question is the biological significance of the E trimer and the E-AII complex. Although the E-AII complex has been found in small quantities in normal plasma (3), these mixed disulfide forms of E have now been found in substantial amounts in Type III dyslipoproteinaemia (3) and AH (this study) probably as a result of disordered lipoprotein metabolism. Although a specific function for apoprotein E has not yet been established, it is possible to speculate that specific receptor sites for E, which have recently been found in fibroblasts and smooth muscle cells (13) may be less sensitive to complexes of apoprotein E thereby protecting particles containing such complexes (such as nascent HDL) from premature removal. Innerarity et al (14) have already shown that binding of HDL containing the E-AII complex is activated after reduction of the disulfide linkages. Alternatively, the combined forms of E apoprotein may have no biological function and only accumulate when E containing lipoprotein particles, are cleared from the circulation at an abnormally slow rate, leading to the polymerisation of apoproteins.

Knowledge of the physiological role of E complexes in lipoproteins may be helpful in identifying the fate of those lipoprotein particles in which they occur. For example, in

our studies with AH subjects, the E-AII and E trimer were mostly (70%) associated with HDL-I (d 1.063-1.080) and the E-AII complex found in HDL of normal plasma (3), was also associated with the HDL-I fraction. The role of E or E complexes and their interconversion may help to establish whether these HDL-I (or HDL_C) particles are newly synthesized, or remnant particles produced during the catabolism of other lipoproteins.

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